A MICROSPORIDIAN INFECTION OF THE DIGESTIVE TRACT OF THE WINTER FLOUNDER, PSEUDOPLEURONECTES AMERICANUS [1]

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Linton (1901) reported sporozoan infections in two small winter flounders, *Pseudopleuronectes americanus,* taken from Katama Bay, Martha's Vineyard, and examined at Woods Hole on August 28, 1900. His account, although brief, is adequate for recognition of the parasite and reads (p. 487), "The walls of the intestine of one throughout almost the entire length and of the other for a short distance were completely covered with sporocysts. The cysts were irregular where crowded together; where not crowded together, which was in but few places, they were elliptical or spherical, of various sizes, but comparatively few reaching 1 mm. in diameter and none much exceeding that. Spores oblong-ovate about 0.003 mm. in length and 0.0015 mm. in diameter. Intestine where affected was chalky-white in color." The accompanying figure shows a "Piece of intestine of *Pseudopleuronectes americanus,* serous coat covered with cysts due to sporosperms (*sic*)." There was no attempt at identification of the parasite, but Linton recognized that it was distinct from another, reported in the same publication (pp. 438 and 439), found in the muscles of the back and sides of the herring, *Clupea harengus,* and the alewife, *Pomolobus pseudoharengus.*

The latter species was identified as a myxosporidian and almost one-half of the young fishes were infected. Tyzzer (1900) reported the discovery and prevalence of this infection in young *P. pseudoharengus;* Auerbach (1910) assigned the species to the genus *Chloromyxum* Mingazzini, 1890; and Hahn (1917) proposed the specific name, *Chloromyxum clupeidae.* The allocation to *Chloromyxum* was based on the spore, which has a quadrilateral apical end and bears four polar capsules. Kudo (1920, p. 94) examined the slides prepared by Tyzzer, and others made from various species of fishes, and reported the infection in *Clupea harengus, Pomolobus pseudoharengus, P. aestivalis, P. mediocris, Brevoortia tyrannus, Stenotomus chrysops,* and *Tautogolabrus adspersus,* taken at Woods Hole. The parasites from the muscles of these fishes were regarded as specifically identical and referred to *Chloromyxum clupeidae* Hahn, 1917.

A third sporozoan was reported by Linton (1901; p. 455) from the liver of the butterfish, *Poronotus triacanthus* (syn. *Rhombus triacanthus*). The cyst was white and globular, about 1.5 mm. in diameter; when compressed it liberated immense numbers of spores, often aggregated in globular or oblong clusters, as large as 0.02 mm. in diameter. The spores were short and thick, with bluntly rounded ends, about 0.0025 mm. in length and a little less than that in breadth and thickness.

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The parasite is obviously a microsporidian and Woodcock (1904; p. 54) regarded it as a species of *Pleistophora*.

A fourth sporozoan was observed by Linton (1901; p. 433): an enormous number of small elliptical bodies, 14 by 6 microns, were found in the intestinal contents of a sting-ray, *Dasyatis centroura*. Since the spores were in the lumen of the gut, it is apparent that they were ingested in food and were parasitic in some animal other than the ray.

The parasites reported by Linton from the wall of the digestive tract of *P. americanus* are similar to and possibly identical with others reported about the same time from flat-fishes of Europe. Hagenmüller (1899) observed the infection in at least one-half ("18 fois sur 30") of the small fishes, *Flexus passer* Moreau (= *Pleuronectes passer*) from littoral pools in the area of Endoûme, Bouches-du-Rhône, France. The parasite was named *Nosema stephani* in honor of M. Pierre Stephan, who first found the cysts and called them to the attention of the author. He wrote (p. 837, "Cette Myxosporidie appartient au genre *Glugea* Thélohan, aujourd'hui *Nosema;* elle infeste, sous forme d'infiltration diffuse ou de kystes, les parois du tube digestif . . . L'infiltration diffuse représente plus particulièrement un mode de pullulation endogène, tandis que les kystes assurent la dissémination du parasite à l'extérieur. Kystes et amas d'infiltration s'observent depuis la partie supérieure de l'oesophage jusqu' à l'extrémeté du rectum, logés dans les tissus ou simplement recouverts par le péritoine. Il n'existe ni amas ni kystes dans le parenchyme d'aucun organe, rein, rate, foie, coeur, etc. Cependant, sous le péritoine à la surface du foie et dans les replis péritonéaux où cheminent des vaisseaux, les kystes sont assez nombreux; j'en ai trouvé jusque sur le conduit cholédoque près de son abouchement avec l'intestin. Dans la paroi intestinale, les kystes siègent dans les couches musculaires et surtout dans la couche conjonctive. J'en ai vu jusque dans la charpente conjonctive des replis de la muqueuse et des villosités, mais jamais, non plus que d'infiltration diffuse, dans la couche épithéliale de l'intestin.

"Ces kystes apparaissent à l'oeil nu comme de petits grains d'un blanc de lait, ovoïdes ou plus rarement sphériques, ne dépassant guère 1 mm. en diamètre, n'atteignant même pour la plupart que quelques dixièmes de millimètre, ou moins encore." Hagenmüller discussed the formation of the cyst and concluded that the membranous wall is produced by the host as a reaction to invasion by the parasite.

A similar and presumably identical species was reported by Johnstone (1901) from the plaice, *Pleuronectes platessa*, taken in the Irish Sea along the coast of Lancashire. The author recognized the parasite as a protozoan, probably a sporozoan, but further identification was not attempted. The infection was limited to the digestive tract and the intestine, from the pylorus to the anus, was thickened and superficially looked like a ripe ovary. The external surface was studded with small, round, white, opaque bodies; the internal surface was disposed in irregular, longitudinal folds, covered with projecting, round white bodies; the lumen was reduced and the mucosa often lost; and the wall measured 3 to 4 mm. in thickness. The cysts were about 0.60 mm. in diameter, with a capsule composed of an outer cuticular and an inner fibrous layer. The spores were oval with a maximum length of 0.005 mm. Figures portrayed the gross appearance of the intestine, the structure of the wall, and the form of the spores.

Woodcock (1904) described a second infection in the digestive tract of the plaice, *P. platessa,* taken near Plymouth, England, and discussed the Myxosporidia in flatfishes. For him, the Myxosporidia Bütschli, 1881 were "characterized (a) by the fact that reproduction by spores goes on throughout the growing or 'trophic' period, and (b) by the complicated process of spore-formation and the nature of the spores." The group was, thus, the equivalent of the Neosporidia Schaudinn, 1900 and the Cnidosporidia Doflein, 1901, and included the Microsporidia Balbiana, 1882. Woodcock reviewed the papers by Hagenmüller, Linton, and Johnstone; he noted that the infection reported by Johnstone was a "ripe, well-matured one," extensively distributed, whereas the infection he studied was only a slight and limited one, from a fish that superficially was quite healthy in appearance. In this specimen, the gut showed little oval patches, 1.00 mm. in diameter, usually projecting slightly on the outer, coelomic side together with other small pyriform appendages, 1.50 to 2.00 mm. in length, attached to the gut by the narrow end. These enlargements were all on the side of the gut to which the mesentery was attached, and in which the blood vessels ran. The functional activity of the intestine was not impaired; the mucosa was intact and normal in appearance. Woodcock compared sections made from the lightly infected intestine with others made from material sent by Dr. Johnstone. He discussed endogenous multiplication ("multiplicative reproduction" of Doflein) in young forms, the spread of the infection into neighboring tissue by diffuse infiltration and the formation of cysts and pseudocysts. He stated (p. 57), "Quite probably 'multiplicative reproduction' is, here, simply a separation of the pansporoblast rudiments, as daughter individuals. Indeed the whole nature of diffuse infiltration in *Glugea* seems to me to support this idea. There is no question of the individual parasites attaining size, still less of any continuity of a protoplasmic mass ramifying in and between host's tissue-cells. It is far rather a cell-infection, visible, when ripe, as separate clumps of spores, each formed from, and representing, one pansporoblast, and either still surrounded by a hypertrophied host-cell, or else free, but only owing to the latter's breakdown." The infections reported by Hagenmüller, Linton and Johnstone were referred to the same species, here designated as *Glugea stephani* (Hagenmüller, 1899) Woodcock, 1904.

Stempell (1904) studied the development of *Nosema anomala* Moniez, 1887, a species from the connective tissue in subcutaneous loci and in the gut-wall, liver, and gonads of the fresh-water stickleback, *Gasterosteus aculeatus.* This species had been transferred by Gurley (1893) to the genus, *Glugea* Thélohan, 1891. Stempell noted that recent investigations had disclosed a series of protozoan species in which the life-cycles consisted of a limited period of vegetative, asexual multiplication, after which different, *"gartete"* forms appear, whose further multiplication is conditional on the conjugation or copulation of two individuals. After citing essentials of these investigations he stated (p. 31), "Bedenkt mann dagegen, dass die allgemeinen Grundzüge der Entwicklung, soweit sie sich feststellen liessen, in allen Fällen dieselben sind, so darf man wohl mit Recht schliessen, dass sämtliche beschriebenen Parasitenformen der Spezies *Nosema anomalum* Monz. angehoren. In der Tat, ein treffender Name für eine so variable Spezies!" Accordingly, he returned the species, *anomala,* to the genus *Nosema.* In this species he reported growth of the protoplasmic masses with rapid, asexual multiplication of

nuclei, followed by the differentiation of sporonts, the admitted progenitors of the sexual generation. His account reads (p. 33), "Wir sehen, wie in der enzystierten Parasitenmasse zunächst ein Wachstum des Protoplasmas und eine starke Vermehrung der Kerne auf rein ungeschlechtlichem Wege erfolgt, wie sich aber schon sehr bald aus dieser vegetativen Parasitenmasse die als Vorfahren der Geschlechtsgeneration aufzufassenden Sporonten differenzieren. Nur dadurch unterscheiden sich die vorliegenden Microsporidien und so viele phänozyste Myxosporidien von der Mehrzahl des anderen Sporozoen, dass diese Geschlechtgeneration durch endogene Knospung im Körper der vegetativen Individuen entsteht."

Weissenberg (1911) reported that about 2% of the smelt, *Osmerus eperlanus*, taken from sources near Berlin and from inlets of the Baltic Sea, were infected with a microsporidian parasite, similar to but distinct from *Glugea anomala*, which he described as a new species, *Glugea hertwigi*. He observed no difference in the infections of fishes from fresh and salt water. In a second paper, Weissenberg (1913) reviewed the work of Stempell (1904) and other authors on microsporidian species and reported on the life-cycles of *G. anomala* and *G. hertwigi*. Since the time of Pasteur it has been known that certain microsporidians invade the ovary and penetrate the ova, with hereditary transmission of infection. Stempell described such infected ova, but Weissenberg (1913) declared the evidence was not convincing. To test the matter, he raised sticklebacks, *Gasterosteus aculeatus*, from eggs. When the yolk-sacs were resorbed he fed small copepoda and daphnids but the fishes did not grow. Fine emulsions of spores were added to the water, but no infection resulted. With other fishes raised in aquaria but fed plankton with an abundance of plant and animal food, growth was good and two young sticklebacks daily were fed plankton mixed with an emulsion of spores. Three weeks after the beginning of the experiment, one of the fishes had a *Glugea*-cyst, 300 microns in diameter, on the wall of the throat. This result demonstrates that a fish raised in the laboratory became infected and provides information on the rate of cyst formation. Weissenberg concluded (p. 157), "Wenngleich die oben dargelegten Befunde bezüglich der Entwicklungsprozesse von *Glugea anomala* in zahlreichen Punkten von den Ergebnissen der Voruntersucher abweichen, so gelange ich doch zu der gleichen Gesamtauffassung wie die älteren Autoren, insbesonder Stempell. Auch nach meinen Befunden kommt *Glugea* ein grosser eigener Plasmakörper mit zahlreichen vegetativen Kernen zu. Die ganze Cyste gehört zum Protozoon. Wirtszellen oder hypertrophische Wirtskerne sind am Cystenaufbau nicht beteiligt." In a subsequent paper, Weissenberg (1921) discussed the problem whether or not the large Plasmakörper, with its large vesicular nuclei, is derived from host tissue or is of protozoan origin. After presenting new evidence he concluded (p. 420), "An der Wirtsgewebsableitung des Plasmakörpers und der bläschenförmigen Kerne der *Glugea*-Cysten kann nun nicht mehr gezweifelt werden. Aufgabe künftiger Forschung wird es sein, die Art der phagocytenartigen verstreut im Bindegewebe auftretenden Fischzellen, die somit den Mutterboden für die *Glugea*-Cysten abgeben, genauer zu eruieren."

Meanwhile, Mavor (1915) reported that about 50% of the *Pseudopleuronectes americanus* examined in the summer and autumn of 1910 at Woods Hole, Mass., were infected with *Glugea stephani*. He also found *Osmerus mordax* at Woods Hole frequently infected with a microsporidian, apparently *G. stephani*. These find-

ings are in marked contrast to others made in the summer of 1912, when no infections were found on examination of 82 *P. americanus and* 22 *O. mordax* taken in the region of St. Andrews, New Brunswick. Kudo (1924) suspected that the parasite of *O. mordax* was *Glugea hertwigi.*

Schrader (1921) found 28% to 53% of the smelts, *O. mordax* from lakes in New Hampshire, and 1.5% to 16% of those from the coast of Maine were infected with a species which he identified as *O. hertwigi* Weissenberg, 1911. The intestine was the primary seat of infection although cysts were present in the liver and gonads. The cysts ranged in size from microscopic to 3 mm. in diameter, but were similar in size in each fish. The highest incidence of infection was in immature fishes, about 10 cm. long; adult fishes were rarely parasitized and Schrader predicated that the majority of infected fish die while immature. Unlike *G. anomala, G. hertwigi* was regarded as specific for smelts since other fishes in the same area were not infected. Furthermore, connective tissue and muscles were not infected, which apparently served to distinguish *G. hertwigi* from *G. anomala.*

Reichenow (1929) described *Glugea stephani* from infections of *Pleuronectes limanda* at Helgoland. He found white cysts, 0.5 mm. in diameter, in the submucosa of the intestine and reported (p. 1099), "Die Parasiten bilden zuerst Infektionsherde in der Darmwand, die von Hagenmuller und Woodcock als Zustand diffuser Infiltration (vgl. S. 1046) bezeichnet werden. Die Parasiten haben jedoch keinen interzellulären Sitz, vielmehr befallen sie im Laufe ihrer Vermehrung zahllose benachbarte Zellen (entweder Bindegewebszellen oder vielleicht Leukocyten, die sich an der Infektionsstelle ansammeln). Um den ganzen Herd herum bildet sich eine dicke Bindegewebskapsel, und so entstehen die Cysten, deren Inhalt also in diesem Falle nicht durch eine einzige Riesenzelle, sondern durch viele infizierte Zellen dargestellt wird. Die fertig ausgebildeten Cysten findet man hauptsächlich von ungeheuren Sporenmassen erfüllt, zwischen denen verstreut Zell- und Kernreste vorkommen. Eine paarige Anlage der Sporen, welche die Stellung dieser Art zu der Gattung *Glugea* begründen würde, ist von keinem der Untersucher beschrieben worden. Ich habe in dem von mir beobachteten Falle eher den Eindruck gewonnen, dass die Sporen einzeln entstehen, so dass die Art also zu *Nosema* zu rechnen wäre. Doch wird sich dies erst bei Beobachtung früherer Infektionsstadien, die übersichtlichere Bilder geben, entscheiden lassen. In meinem Falle, in dem die Cysten dicht gedränkt in der Darmwand sassen, war die Schleimhaut auf weite Strecken völlig abgestossen; es ist daher zu vermuten, dass die Fische an starken Infektionen zugrunde gehen."

Recent accounts have added little information on microsporidian infections of fishes. Bond (1938) identified cysts found in sections of the stomach of *Fundulus heteroclitus* taken in Chesapeake Bay as *G. hertwigi,* but the determination may not be correct. Fantham *et al.* (1941) described an infection in the hindgut of a specimen of *O. mordax* taken from Lake Edward, Quebec, and listed the parasite as *G. hertwigi* var. *canadensis.* Also, they reported *G. stephani* in the submucosa of the intestine of *P. americanus* and *Limanda ferruginea,* taken near Halifax, Nova Scotia; *L. ferruginea* was recognized as a new host of the parasite. Haley (1952) described a severe epidemic of microsporidiosis in *O. mordax* in Loon Pond, Gilmanton, New Hampshire, and 16 of 20 *O. mordax* from the Oyster River taken at

Durham, N. H., were infected by the same species, which he identified as *G. hertwigi*.

The Microsporidia are chiefly parasites of invertebrates, especially crustaceans and insects. The classification of the Microsporidia or Microspirida is based primarily upon the form and structure of the spores and to a lesser degree upon differences in the details of sporogenesis. The parasites of *P. americanus* belong to the family Nosematidae, characterized by small, oval or ovate spores, each with one polar filament. The genera are distinguished by the number of spores that are produced by each sporont. According to Poisson (1953), in *Nosema* each sporont develops into a sporoblast and produces a single spore; in other genera the numbers of spores produced are: *Glugea* Thélohan, 1891 and *Pérezia* Léger et Duboscq, 1909, two spores; in *Gurleya* Doflein, 1898 and *Pyrotheca* Hesse, 1935, four sporoblasts and four spores; but in *Stempellia* Léger et Hesse, 1910, the numbers of spores produced are: *Glugea* Thélohan, 1891 and *Pérezia* Léger et Duboscq, number varies from 8 to 32; and in *Plistophora* Gurley, 1893, each sporont (pansporoblast) produces more than 16 spores. It is generally believed that the microsporidia are narrowly host-specific. According to Poisson (1953) some 40 species in the genera *Plistophora, Glugea,* and *Nosema* occur in fishes and one species, *Glugea danilewsky,* occurs in the muscles and connective tissue of *Rana fusca, Emys orbicularis, Natrix natrix* and other hosts. If this determination is correct, the distribution of *G. danilewsky* belies the opinion that species of *Glugea* are host-specific.

The life-history of the Microsporidia, as conceived by Debaisieux (1928), comprises two distinct phases: a multiplicative stage, schizogony, beginning with the liberation of the uninucleate or binucleate sporoplasm or planont from the spore and its entry into a host-cell, and sporogony, a spore-forming stage, in which sporonts produce sporoblasts that give rise to resistant spores, the infective agents that serve for dispersal of the parasite and the infection of new hosts. According to Kudo (1924, p. 34), "No intermediate host animals have up to date been found for Microsporidia. The infection of a new host animal takes place when the latter ingests spores of a specific microsporidian capable of germinating in its gut." A similar statement was made by Dogiel, Petrushevski and Polyanski (1961) but no reference to experimental evidence was cited.

It is generally agreed that the life-cycle of the microsporidian involves sexual phenomena but there is wide disagreement concerning the location in the cycle where meiosis and syngamy occur. Meiotic phenomena have never been observed in the Microsporidia and syngamy has been reported by autogamy of nuclei in the sporoplasm before or after release from the spore, and also by nuclear fusion preceding sporont formation. Writing on sexual phenomena in Protozoa, Hall (1953, p. 80) stated, "A reduction of the chromosomes to the haploid number may occur in gametogenesis (*gametic meiosis*), in an early division of the zygote (*zygotic meiosis*), or in one of the preganic divisions in conjugation (*conjugated meiosis*). The type of meiosis varies in different Protozoa. Available data indicate that the Heliozoida, Foraminifera, Cnidosporidia, and Ciliophora are diploid throughout most of the life-cycle." An opposite opinion was stated by Cheissin and Poljansky (1963, p. 343), "In the life-cycles of the Sporozoa the alternation of sexual process and sporogony or that of sexual process, sporogony and repeated asexual multipli-

cation by means of schizogony occurs. All the developmental stages but zygote are haploid ones because the meiosis usually appears during the process of sporogony followed by formation of sporozoites." The statement by the Russian authors apparently is based on the situation in the malarial parasites, but the Microsporidia are distinct from the Haemosporidia and the life-cycles may be quite different. Indeed, Kudo (1944, p. 50) reporting on the life-cycle of *Nosema notabilis* Kudo, 1939, stated, "Schizogony is by binary fission. No sexual process has been observed in the development of *Nosema notabilis*."

The small size of the amoeboid stages and of the spores, usually less than 4 microns in length, together with the inability to obtain early stages by controlled experimental infections of fishes, has made it impossible to describe the developmental cycle of these microsporidian species with assurance. The time and place of chromosome-reduction in meiosis and of syngamy are controversial. Cells with two nuclei may represent a stage before fusion of gametes or the first division of a zygote. Specific distinctions are often precarious and even generic diagnoses are unsatisfactory. In his monographic treatise, Poisson (1953) stated (p. 1043), "Mais bien des espèces de Microsporidies sont insuffisamment étudiées; trop d'espèces ont été décrites comme nouvelles parce qu'elles étaient trouvées dans des hôtes nouveaux. D'après Steinhaus et Hughes par example, *Nosema destructor* S. et H. a été observée chez au moins 10 espèces d'Insectes apparentant à trois groupes différents: la chenille de *Gnorimoschema operculella* (Zeller) (Lépidoptère), des Hyménoptères, des Névroptéroides. Il est donc des Microsporidies qui ne manifestent qu'une spécificité toute relative. D'autre part, les caractères distinctifs utilisés pour séparer les espèces, et même les genres, n'offrent peut-être toujours la précision désirable et certains genres, tels les genres *Nosema, Plistophora, Glugea, Perezia,* devront être revisés."

Microsporidian infections of *Pseudopleuronectes americanus* have long been known by members of the staffs of the New York Aquarium and the New York State Conservation Department, but precise and detailed records of incidence and intensity are not available. Dr. Ross F. Nigrelli, at the Aquarium of the New York Zoological Society, has observed the frequent occurrence of the parasite in fishes of the New York area and Mr. John C. Poole of the Conservation Department reports that the infection has a "spotty" distribution, *e.g.,* in one year over 25% of the young of that year taken in Shinnecock Bay were infected and no infection was found in the same location the following year.

Materials and Methods

The present investigation was begun in the summer of 1961 and has been conducted more or less continuously since that time. Over 1000 fishes, *P. americanus*, taken from different locations in New England, have been inspected for microsporidian infection. Data have been compiled (Tables I–VI) on the number of fishes examined, the time of year and area where they were caught, their size, sex, and the incidence and intensity of infection. Fishes taken on Georges Bank, off Yarmouth, Nova Scotia, were caught on the August, 1963, cruise and those from Nantucket shoals on the April, 1964, cruise of the Albatross IV. Records denote the organs involved and the extent of infection. Winter flounder are present from April to November in Woods Hole harbor. The stomachs and intestines of 751

TABLE I

Incidence of microsporidian infection in winter flounder from Woods Hole Harbor in 1962

Month	Number examined	Number infected	Per cent infection
April	80	3	3.4
May	67	2	2.3
June	86	1	1.2
July	102	7	6.9
August	156	6	3.8
September	86	0	0.0
October	125	3	2.4
November	49	4	8.2
Total	751	26	3.5

fishes were removed and preserved for food analysis, and patent sporozoan infections were noted. Very light infections may have been missed, so the recorded intensity is minimal. Analysis of the stomach-contents was made to determine the kinds and amounts of food ingested. In November, 1964, about 300 young fishes that measured from 40 to 110 mm. in length were taken in Lake Tashmoo, Martha's Vineyard, where the infection-rate was known to be high. Eighty-five of these fishes, which died at the time of collecting or a few hours later, were examined and the results are given in Table VI. Heavy infections included those where the infil-

TABLE II

Length-distribution of microsporidian-infected winter flounder compared with that of all winter flounder examined, in Woods Hole Harbor during 1962

Length (cm.)	Number of fish		Length (cm.)	Number of fish	
	Total	Infected		Total	Infected
12	1		28	64	6
13	1		29	70	
14	1		30	66	2
15	6		31	70	1
16	3		32	66	1
17	1		33	48	2
18	7	1	34	45	1
19	11	1	35	33	2
20	11		36	20	
21	7		37	16	
22	15		38	6	1
23	19	2	39	6	
24	18		40	1	
25	33	2	41	2	
26	48	2	42	2	
27	51	2			
				751	26

TABLE III

Mean weights of infected and non-infected winter flounder from Woods Hole Harbor in 1962

Mean length cm.	Infected		Non-infected	
	Number	Mean weight	Number	Mean weight
July–August				
males				
23	2	193	5	211
30	2	366	9	351
33	1	443	7	439
34	1	402	4	513
females				
18	1	76	—	—
23	1	150	3	165
27	1	232	6	272
28	4	278	10	293
October–November				
males				
19	1	70	1	64
26	2	194	6	223
females				
28	1	280	9	270
31	1	404	9	368
35	1	541	8	551
38	1	652	3	774

tration was massive and the gut was partly or largely destroyed; light infections included those with from one to 20 cysts in the wall of the intestine. By the time that cysts are formed the infection is already well established.

In Table VI, the winter flounders less than 100 mm. in total length were of the 1964 year class, *i.e.*, less than a year old. Those of 100 mm. or more in length probably were of the 1963 year class, but final age-determination was not made.

Since the microsporidiosis is located primarily in the intestine, and the infective agent was presumably taken in with food, the stomach-contents of 751 fishes from

TABLE IV

Amount of food in stomachs of infected and non-infected winter flounder from Woods Hole Harbor in July–August, 1962

Month	Infected			Non-infected		
	Number	Mean length cm.	Food grams	Number	Mean length cm.	Food grams
July	7	28	1.25	102	30	2.19
August	6	26	1.02	156	27	0.82

TABLE V

Incidence of microsporidian infection in winter flounder from different New England fishing grounds

Location	Georges Bank		Off Yarmouth, Nova Scotia		Nantucket Shoals		Off Plymouth, Mass.	
Length cm.	Total number	Number infected	Total number	Number infected	Total number	Number infected	Total number	Number infected
11–15	1	0	0	0	0	0	0	0
16–20	1	0	1	1	0	0	0	0
21–25	0	0	0	0	16	1	1	0
26–30	1	0	0	0	25	1	0	0
31–35	6	0	2	0	53	11	12	1
36–40	6	0	3	0	23	7	5	2
41–45	16	0	2	0	8	1	1	0
46–50	2	0	0	0	1	0	0	0
51–55	3	0	0	0	0	0	0	0
56–60	1	0	0	0	0	0	0	0
61–65	1	0	0	0	0	0	0	0
Total	38	0	8	1	126	21	19	3
Per cent infected	0		1.2		16.7		15.8	

Woods Hole harbor have been examined in an attempt to discern the source or sources of the infective agent or agents. Also, since Microsporidia are presumed to be one-host parasites, microsporidian cysts from winter flounder gut-wall embedded

TABLE VI

Incidence of microsporidian infection in small winter flounder from Lake Tashmoo, Martha's Vineyard

Length (mm.)	Degree of infection		
	Heavy	Light	None
41–45	2	0	0
46–50	4	0	1
51–55	6	1	1
56–60	6	3	3
61–65	2	3	4
66–70	0	2	7
71–75	1	6	8
76–80	1	2	4
81–85	0	2	4
86–90	1	4	1
91–95	0	0	2
96–100	0	0	0
101–105	0	0	2
106–110	0	0	2
Total	23	23	39

Per cent infected, 54.1.

in pieces of clam, *Mercenaria mercenaria,* have been fed to other winter flounder kept in aquaria in attempts to induce experimental infections. Cysts were fed to two fish in the summer of 1962. They were examined six weeks later and there was no evidence of infection. Five fish were fed cysts in November, 1964. Three were examined in April, 1965, and the other two were autopsied in June, 1965. No infection resulted from these experiments. It appears that direct infection does not occur, that either the sporoplasms do not emerge from the spores or they fail to invade the intestinal epithelium.

Tissues from natural infections were fixed in different fluids, cut in serial sections at 5 and 10 microns in thickness, and stained for particular effects. Haematoxylin and erythrosin were used for general purposes and routine pathological staining. Heidenhain's iron technique was employed on thin sections for cytological details and azan trichrome for special histology.

Results

The incidence of infection in 751 fishes taken in the Woods Hole harbor and examined each month, April through November, 1962, and data on the size and sex of the fishes are presented in Tables I–III. There was no apparent effect of seasonal or sexual differences. Table IV records the amount of food in the stomachs of infected and non-infected fishes of comparable sizes taken from Woods Hole harbor in July and August, 1962. There was no obvious relationship between infection and amount of food in the stomach. Stomach-contents of 386 fishes collected in weekly samples in September, October, and November, 1961, consisted by weight of algae, 42%; mollusks, 25%; polychaetes, 24%; crustaceans, 5%; and other (mostly unidentified), 4%. The results of analyses made in 1962 are similar, with less algae eaten in the spring and summer. No fish were found in any of the flounders examined, thus confirming the statements by Bean (1903), Breder (1929) and Bigelow and Schroeder (1953) that young flounders feed exclusively on algae and invertebrates, chiefly crustaceans and polychaete annelids. The account of Bigelow and Schroeder is very complete and includes the findings of Breder and Linton as well as their own observations. It is generally agreed that the small mouths of these flounders preclude the ingestion of fishes as food, and it appears certain that fish are of no significance in the diet of *P. americanus* in the Woods Hole area.

The incidence of infection in winter flounder of different sizes taken from different coastal areas and from Georges Bank, which is offshore, is presented in Table V. Although the number of fishes from Georges Bank is small, the absence of infection there may be significant. There is evidence that Georges Bank winter flounder are geographically isolated from those on inshore grounds and that they have no contact with the shore at any time during their lives. Results from the release on inshore grounds of over 10,000 tagged winter flounder indicate that only one was re-caught on Georges Bank (Perlmutter, 1947; Bigelow and Schroeder, 1953). Perlmutter also reported that winter flounder from Georges Bank have more fin rays than those from inshore grounds north and south of Cape Cod. Winter flounder from inshore subpopulations, on the other hand, are closely associated with the shore, spending their first year in estuaries and bays where much of the spawning occurs, and where infection may take place.

The data from the small fishes taken in November, 1964, at Martha's Vineyard (Table VI) are particularly interesting. In addition to the information presented in the table, 49 individuals in the same size-range died in the period November 20 to December 7, 1964. Nineteen of these fishes, mostly 65 to 85 mm. in length, were infected, with an incidence of 38.8%. Seven of the infections were heavy; 12 of them were light. Fifteen of the remaining fishes were killed March 12, 1965, and 8 of them, *i.e.*, 53.3%, were infected. Three of these infections were heavy; five were light. Inspection of the data from the 149 fishes examined shows that infection was greatest in small fishes. Almost all of the heavily infected ones were less than 80 mm. in length and some of them were less than 50 mm. in length. Since development of such massive infections must take some time, it is apparent that infection occurred very early in life, when the food consisted of small invertebrates. Comparison of the findings recorded in Tables II and VI, indicates strongly that fishes heavily infected during the first year of life do not survive into their second year.

The site of infection is primarily the wall of the intestine and pyloric ceca, but in moderate and heavy infections, other structures adjacent to or in contact with the gut may be involved. These include the bile-duct, liver, mesenterial lymph-nodes and the ovary. The infections observed were already well advanced and were manifest by cysts (Figs. 4, 5) embedded in the connective tissue of the organs affected. In larger fishes, most of the infections were light and apparently did not seriously affect the hosts. In light infections, the cysts were usually on the external wall of the intestine, but in heavy ones, the gut wall was largely supplanted by layers of cysts. In such instances the intestine had a chalk-white, pebbled appearance and the wall was rigid, thickened and hard. Photographs of intact normal and parasitized digestive tracts and of sections of the intestine and cecum of infected fishes portray the effects of massive infection. Figure 1 is of a normal digestive tract. In the specimen shown in Figure 2, the anterior end of the digestive tract is the principal site of infection, whereas in Figure 3, it is the rectal end of the specimen that is most heavily parasitized. Figure 4 is a photograph of a cross-section of the intestine shown in Figure 2, and Figure 5 is a photograph of a cross-section of one of the pyloric ceca taken from the specimen shown in Figure 2. The epithelium of the intestine in Figure 4 is denuded and the lumen of the cecum (Fig. 5) is almost occluded.

The cysts are spherical to oval unless deformed by pressure. They measure 0.6 to 1.0 mm. in diameter and the wall (Figs. 6, 7) is composed of laminated layers that have the structural appearance and staining reactions of the connective tissue of the host. In addition to those in the cysts, there are masses or strands of spores scattered about in the tissue of the gut wall, often associated with or paralleling blood vessels. The material at present available for study consists of relatively mature infections and the multiplicative phases have largely been completed. Rarely, near the wall of a cyst or in the intercystal areas there is a cell, which may be a pansporoblast, which contains a large number of bodies that color deeply with nuclear stains. Whether or not these structures are the nuclei of sporoblasts could not be determined. Below the connective capsule of the cyst there is often a narrow layer of stainable material, termed endoplasm by Woodcock, which contains large oval, apparently pycnotic, nuclei with fragmented chromatin and distinct nu-

cleoli. Their presence suggests that the cyst is formed around a number of host-cells, whose cytoplasm has been consumed and whose nuclei persist below the wall of the cyst. The spores are oval to ovate, and when fixed and stained measure about 4 by 2.5 microns. Precise and accurate measurements of such minute and refractive bodies are difficult. The basal, wider end of the spore contains a vesicle

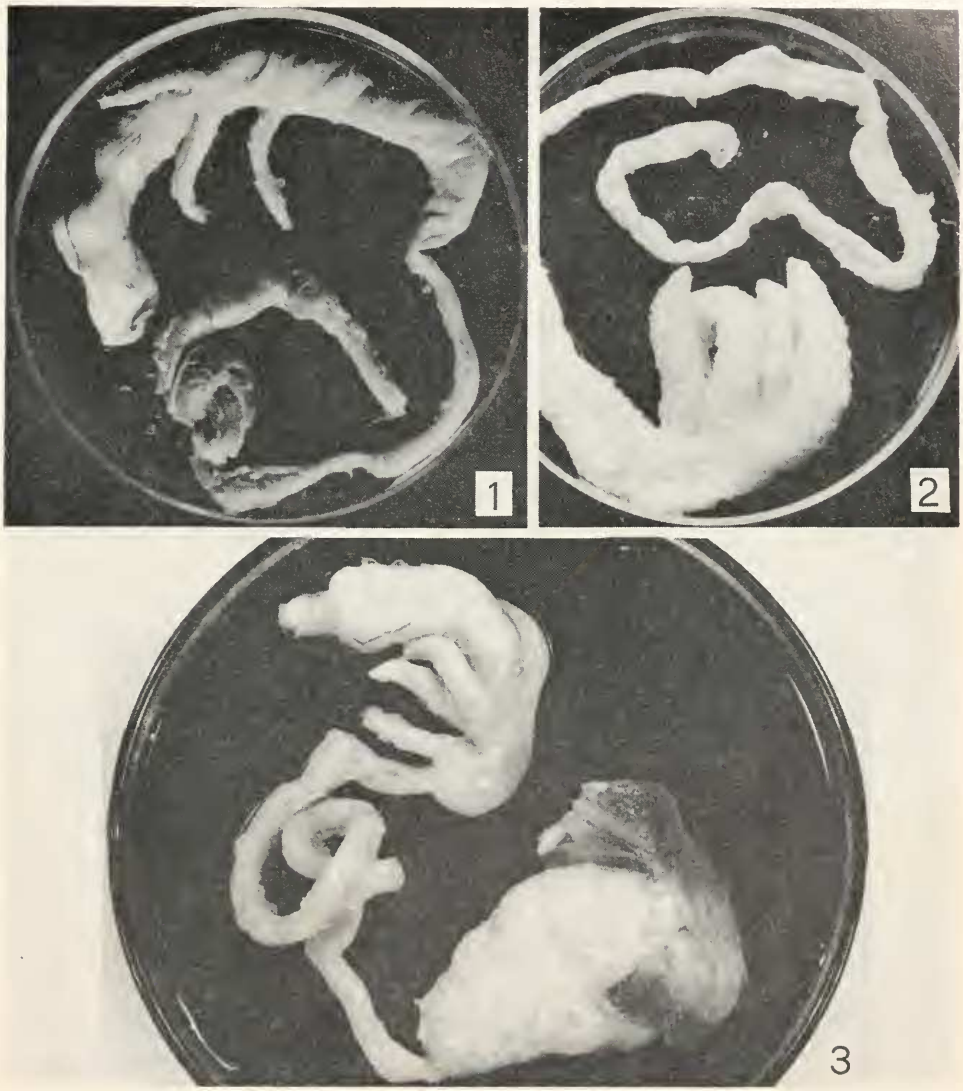

PLATE I

FIGURE 1. Digestive tract of *P. americanus,* normal condition, in a Petri-dish, 9.5 cm. outside diameter.

FIGURE 2. Pyloric ceca and intestine of infected fish, same magnification as Figure 1.

FIGURE 3. Digestive tract of infected fish, same magnification as Figure 1.

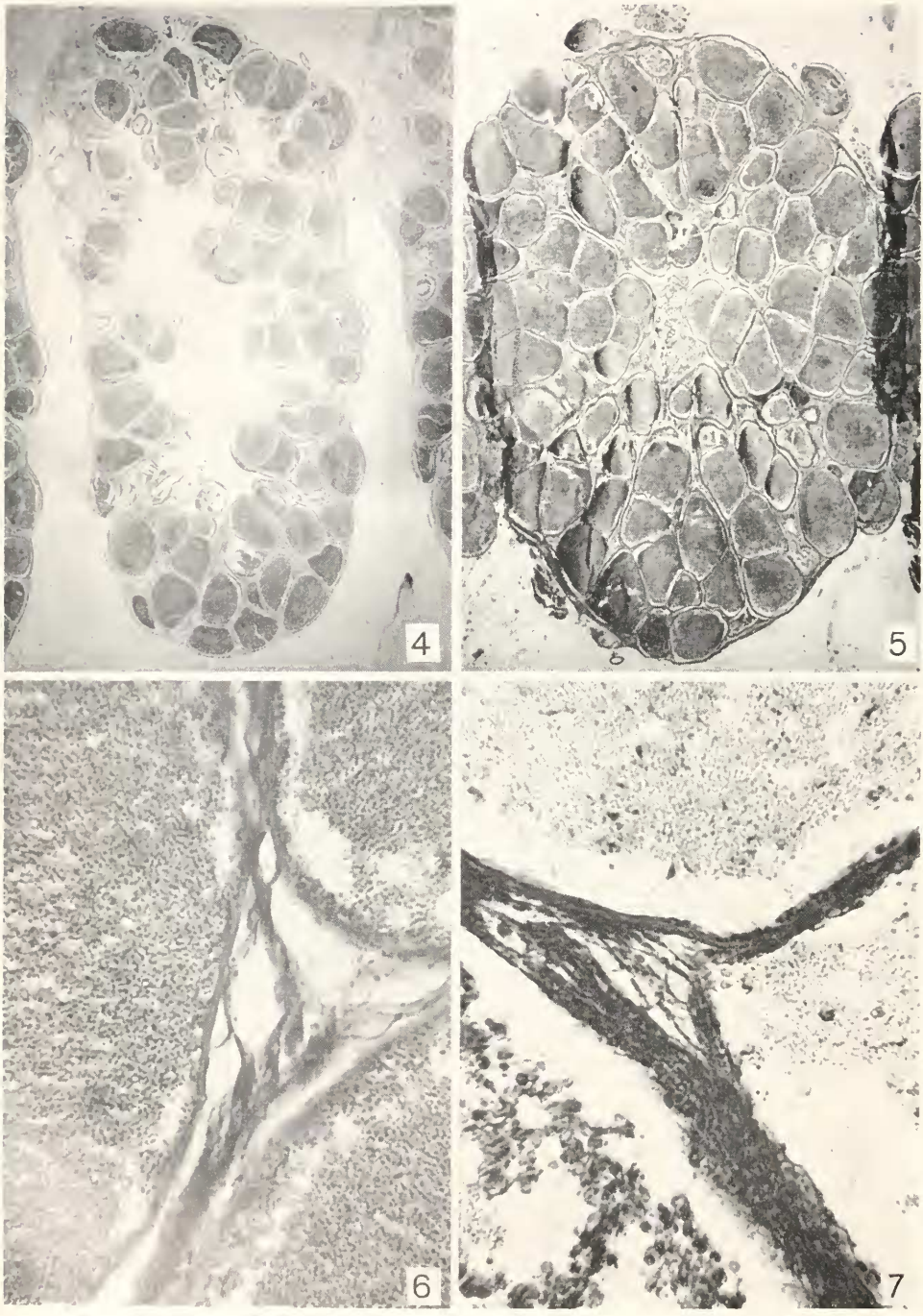

PLATE II

that may occupy almost one-half the length of the spore. The apical end also contains a smaller vesicle, while the central portion contains a band of chromatic material, often in the form of strings of particles or granules, and a single strand extends to the apical end of the spore.

Discussion

The most comprehensive account of the Microsporidia is the monograph by Kudo (1924). He gave a review of morphology and life-cycles, with a description and taxonomic survey of all previously described species. In a later study, Kudo (1944) stated (p. 38), "The early phases of the development of Microsporidia have not been seen in many species. In a few instances of experimental infection, certain portions of the development have been seen, but in no case has observation in life been carried through." It is generally agreed, however, that the life-cycle of a microsporidian species consists of two distinct phases or stages: a multiplicative stage, schizogony, and a spore-forming stage, sporogony. In the multiplicative phase, cell division is rapid and according to certain investigators it may result from binary fission following nuclear division or multiple fission if nuclear division is rapid and cytoplasmic division is delayed. It has been suggested (Kudo, 1924) that in certain species the schizonts (meronts of Stempell, 1902) are not motile and as a consequence that the progeny of a sporoplasm remains in the host-cell and that all the spores formed in that cell are derived from the initial parasite. But usually the infection is invasive, with diffuse infiltration of tissue, and such a condition could result from either the penetration and dispersal of enormous numbers of planonts or by the liberation of schizonts and their ingestion by leukocytes or macrophages which accumulate at sites of inflammation and which could transport the schizonts to other areas and extend the infection. At the end of the schizogonic phase, sporonts are formed but the factors involved and details of the phenomena which result in the formation of sporonts are equivocal. Supporting the observations of Weissenberg (1914), Debaisieux (1920) and Guyénot and Naville (1922), Kudo (1946, p. 162) stated, "In the Microsporidia, autogamy appears to initiate the spore-formation at the end of schizogonic activity."

In the present study, the inability to obtain experimental infection of fishes has precluded observations on the multiplicative phases of the life-cycle. But this inability has raised important and perplexing problems. Since fishes become infected when only 50 mm. in length and when the food consists of small invertebrates, it seems probable that a second or intermediate host may be required in the life-cycle of the parasite. Such an invertebrate may be merely a paratenic or transport host, which ingests spores from a dead fish and is then eaten by a small flounder, or it may be essential in the completion of the life-cycle of the parasite. Since

Figure 4. Photomicrograph of cross-section of the intestine shown in Figure 2. Note lack of digestive epithelium and disintegration of the gut wall.

Figure 5. Photomicrograph of cross-section of one of the pyloric ceca shown in Figure 2. The infection is more intense in this area than in the intestine.

Figure 6. Photomicrograph of section of pyloric cecum, greater magnification, to show connective tissue capsular wall of the cyst and number of spores.

Figure 7. Photomicrograph of section of pyloric cecum, showing walls of cysts and adjacent nuclei and cells.

small crustaceans are carnivorous and constitute a considerable part of the food of small fishes, they become suspect. According to the account of Frederick E. Smith, (The Benthos of Block Island Sound: Ph.D. thesis, Yale University, 1950, 213 pp. and appendices), 75% of the food of the winter flounder consisted of amphipods and 43% of the amphipods were *Leptocheirus pinguis*.

Other questions also arise: why are certain infections mild while others become massive? Do older fishes develop resistance to infection and restrict the invasive activity of the parasite? Why do the cysts manifest such uniformity in size? Finally, in view of the statements of Reichenow (1929) and Poisson (1953) that generic concepts are tenuous, what is the status of *Glugea* and does the species, *stephani*, belong in that genus? The answers to these and other questions await further investigation on the life-cycle of the species.

Summary

A microsporidian infection of the blackback or winter flounder, *Pseudopleuronectes americanus*, has been investigated. It was first noted at Woods Hole, Massachusetts by Linton (1901) and may be identical with similar infections of European flounders reported by Hagenmüller (1899), who described the parasite as *Nosema stephani*. Woodcock (1904) transferred the species to *Glugea*, a genus erected by Thélohan (1891) to contain a parasite of the striated muscle in *Cottus scorpio* and *Callionymus lyra*, which he described as a new species, *Glugea microspora*. Gurley (1893) predicated that *G. microspora* is identical with *Nosema anomala* (Moniez, 1877), although he recognized *Glugea* as a valid genus, distinct from *Nosema*. In New England the infection is common in *P. americanus*. The incidence and intensity of infection in fishes of different sizes and from different geographical regions are reported, together with an account of the resultant pathology. Attempts to obtain experimental infection of fishes have not been successful and the life-cycle of the parasite remains unknown.

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